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Heat-Shock Protein Expression in *Mytilus californianus:* Acclimatization (Seasonal and Tidal-Height Comparisons) and Acclimation Effects

DEIRDRE A. ROBERTS, GRETCHEN E. HOFMANN[1], AND GEORGE N. SOMERO[2]

Department of Zoology, Oregon State University, Corvallis, Oregon 97331-2914

Abstract. Heat-shock protein (hsp) expression was examined in gill of field-acclimatized and laboratory-acclimated mussels (*Mytilus californianus*) from the Oregon coast. Endogenous levels of heat-shock proteins in the 70-kDa class (hsp70 isoforms) and profiles of induction temperature for newly synthesized hsp70 were measured in freshly field-collected specimens as functions of location height in the intertidal and season, and in mussels after 7 weeks of laboratory thermal acclimation. There were significant differences in endogenous levels of hsp70 as functions of season and collection height. Strong induction of new hsp70 synthesis occurred at body temperatures within the range measured in field specimens. Profiles of hsp70 thermal induction varied significantly with season, but not with height of collection. In contrast to the large differences in hsp70 expression between winter- and summer-acclimatized mussels, no differences related to temperature occurred in the differently acclimated mussels. The differences found between the effects of field acclimatization and laboratory thermal acclimation suggest that the stress response is modulated by environmental factors in addition to body temperature. Thus, caution is required in extrapolating from laboratory acclimation studies to acclimatization effects in field populations. The seasonal and tidal-height variations in the heat-shock response are discussed in the context of energy costs of protein turnover.

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[1] Direct correspondence to Dr. Hofmann at the Department of Biology, University of New Mexico, Albuquerque, NM 87131-1091.

[2] Current address: Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950-3094.

Introduction

All but one species so examined have been found to synthesize heat-shock proteins (hsps) in response to exposure to temperatures of a few to several degrees Celsius above those normally experienced by the organism (Craig, 1985; Bosch *et al.*, 1988; Welch, 1993; Parsell and Lindquist, 1993; Becker and Craig, 1994). At temperatures near the upper limit of thermal tolerance, hsps may be the major, and in some cases the only, proteins synthesized (Morimoto *et al.*, 1990). The presence of the heat-shock response across taxa suggests an ancient origin for the response (Gupta and Singh, 1992). In addition, many studies suggest that hsps play a critical role in thermal tolerance at the cellular level (for review see Parsell and Lindquist, 1994). The recognition that some hsps are members of a broad class of proteins termed molecular chaperones—proteins that prevent improper aggregation of structurally non-native proteins and assist in correct protein folding and compartmentalization—has helped to clarify the potential roles of hsps in response to heat stress. Thus, hsps prevent aggregation of heat-damaged proteins in the cell, and therefore may indirectly assist in restoring the native structure of proteins that are reversibly damaged by high temperatures (Wiech *et al.*, 1992; Jakob *et al.*, 1993; Parsell and Lindquist, 1993; Becker and Craig, 1994; Hartl, 1996).

Although major advances have been made using isolated cell lines and unicellular organisms to resolve the mechanisms by which molecular chaperones function and by which their synthesis is regulated, relatively few studies have examined the heat-shock response in organisms in their natural habitats, in which thermal stress may greatly vary in time and space. Studies of the heat-

shock response in natural populations are important for several reasons, including (1) determining what temperatures are in fact sufficiently high to induce the response under natural habitat conditions; (2) characterizing the plasticity of the response (*e.g.*, changes in induction temperatures and endogenous levels of hsps) in concert with variation in seasonal thermal regimes; and (3) establishing the relative magnitude of the heat-shock response in conspecifics exposed to different thermal regimes in their distinct microhabitats. In view of the high fraction of metabolism directed to protein synthesis and protein turnover (Hawkins and Bayne, 1992), all of this information could be useful in developing models of ecological energetics—for example, in understanding the energy cost of existence in the face of fluctuating temperatures. Further understanding of the heat-shock response could be especially important in developing and refining conceptual models in community ecology. These would include models of environmental stress, distribution and zonation, and diversity gradients, which invoke the importance of sublethal abiotic stress in determining organisms' distribution limits, competitive relationships, and life-history strategies (Menge and Olson, 1990; Bertness and Callaway, 1994).

As part of a broad study of the physiological ecology of temperate, rocky intertidal invertebrates, we have examined several attributes of the heat-shock response in the California mussel, *Mytilus californianus*. The rocky intertidal zone exposes its inhabitants, especially sessile species like *M. californianus*, to wide ranges of temperature, as well as to desiccating conditions, wave-exposure stress, variations in access to oxygen and nutrients, and high levels of UV radiation (Newell, 1979). Variation in physical condition occurs both seasonally and as a function of height in the intertidal zone. In general, the high-intertidal zone is characterized by more extreme abiotic conditions than the low-intertidal zone, due to wider fluctuations in temperature and greater exposure to aerial conditions during tidal cycles.

Mytilus californianus is an appropriate species for studies of natural variation in the heat-shock response with season and microhabitat location of conspecifics. *Mytilus* spp. are a prominent component of many temperate, wave-swept rocky shores (Seed and Suchanek, 1992). *Mytilus californianus* occurs along the Pacific coast of North America from Alaska to Baja California (Morris *et al.*, 1980), and the upper and lower limits of its beds typically define the boundaries of the mid-intertidal zone (Suchanek, 1978). The lower limit of the *M. californianus* zone is primarily determined by biotic interactions, predation and competition (Paine, 1966, 1974), whereas the upper limit is generally considered to be most influenced by physical constraints involving temperature and desiccation (Seed and Suchanek, 1992). Harger (1970) found that *M. californianus* at higher tidal heights had decreased growth rates and attained smaller maximal sizes than conspecifics found lower in the intertidal zone.

We examined the heat-shock response in *Mytilus californianus* to determine how it varies with season and with microhabitat location. Two characteristics of the response were studied: the temperatures at which hsp induction occurs and the endogenous levels of hsps. We focused on hsps of the 70-kDa size class (*i.e.*, hsp70 isoforms) and found that both attributes of the heat-shock response change significantly with season, and that the concentration of hsp70 in high- and low-intertidal individuals of *M. californianus* also differs significantly. We performed a companion laboratory study of thermal acclimation to determine whether temperature *per se*, independent of other environmental factors like aerial exposure, influenced variations in the heat-shock response. We discuss these results in the context of the expression of heat-shock proteins in organisms under ecologically relevant habitat conditions and in relation to how laboratory acclimation studies may obscure the complete range of expression patterns found in field-acclimatized organisms.

Materials and Methods

Acclimatization studies: field collections and body-temperature measurements

Mytilus californianus (shell length 60–80 mm) was collected at Strawberry Hill on the central Oregon coast (44° 15′N, 127° 07′W) in July of 1993, and in February, March, May, June, and August of 1994. Collections were performed on a single day each month. In February, mussels were collected from a mid-intertidal site. In all other months, mussels were collected from low- and high-intertidal sites corresponding to the lower and upper limits of the *M. californianus* zone at Strawberry Hill. For the heat-shock induction experiments, freshly collected mussels were transported to the laboratory in ambient-temperature seawater (8° to 12°C) within 4–6 h of collection. In the laboratory, mussels were kept at 10°C in tanks of recirculating seawater and used in hsp induction experiments within 1–3 days of collection.

Samples of gill tissue for solid-phase immunochemical quantification of hsp70 levels (western blotting) were collected in July 1993 and February 1994. In the field, gill lamellae were dissected from mussels gathered at the high- and low-intertidal sites at the beginning and end of the emersion period, during the more extreme low tide on the collection day. The tissue samples were immediately frozen on aluminum blocks chilled on dry ice, transported to the laboratory on dry ice, and stored at −70°C until processed for protein electrophoresis and western blotting.

The body temperatures of mussels at the field collec-

tion sites were measured with a thermocouple connected to a hand-held digital thermometer (Omega Inc.). The probe was inserted into a small hole drilled through a valve, and temperatures were recorded at intervals of about 20 min throughout the emersion period. Between measurements, the holes were plugged with modeling clay to prevent evaporative water loss.

Acclimation studies: field collections and acclimation procedure

Specimens were collected at Strawberry Hill on 15 May 1994, during low tide. Gill-tissue samples for western analysis were collected from mussels dissected in the field, as described above. Mussels for acclimation experiments ($n = 125$) were haphazardly selected from a mid-intertidal rock bench, within an area of about 4 m^2. Mussels were transported to the laboratory in ambient-temperature seawater (12°C) within several hours of collection and immediately placed in a tank with recirculating seawater at 13°C. The next day, the mussels were divided into four tanks, each containing 30 mussels. One tank was maintained at 13°C and the other three tanks were adjusted to one of three temperatures, 10°, 17°, or 20°C, at a rate of 2°C per day. All tanks were maintained for 7 weeks after acclimation temperatures (10°, 13°, 17°, and 20°C) were reached. Mussels were fed an algal concentrate mixture (Algae Preserve Diet 'B'; Coast Seafoods Co., Bellevue, WA) every 4 days.

Heat-shock-protein induction experiments

For the field-acclimatization studies, hsp induction experiments were conducted on freshly collected mussels in February, March, May, June, and August of 1994. For the laboratory-acclimation studies, hsp induction experiments were conducted in May 1994 on freshly field-collected mussels and in July 1994, at the end of the 7-week acclimation period, on mussels acclimated to temperatures of 10°C, 13°C, 17°C and 20°C. All induction experiments were conducted with gill tissue because of the ease with which several similar fragments of this tissue could be obtained from each individual, and because of the ability of gill to take up dissolved amino acids from the medium. Whole gill lamellae were dissected from mussels held in chilled seawater. The lamellae were cut into small fragments weighing about 200 mg. These fragments, one for each temperature tested, were immediately placed into 500 μl of incubation medium (Hepes-buffered artificial seawater [20 m*M* Hepes, 7.57 m*M* $(NH_4)_2SO_4$, 375 m*M* NaCl, 9.35 m*M* KCl, 2.7 m*M* $NaHCO_3$, 17.95 m*M* Na_2SO_4, 37.7 m*M* $MgCl_2$-$6H_2O$, 8 m*M* $CaCl_2$-$2H_2O$, 10 m*M* glucose]) in 1.5-ml microcentrifuge tubes that had been pre-equilibrated at the heat-exposure temperatures. Immediately prior to the addition of gill, 5 μl of ^{35}S-labeled methionine and cysteine (Trans-35, ICN Radiochemicals) was added to the incubation medium. Gill fragments were radiolabeled for 2 h at the following temperatures: 10°, 13°, 17°, 20°, 23°, 25°, and 28°C. During the incubation period, gill fragments were aerated frequently by blowing a stream of air onto the surface of the incubation medium with sufficient force to stir the medium and oxygenate the buffer. After the incubation period, gill fragments were rinsed in 2 vol of nonradioactive incubation medium and then either frozen immediately on dry ice or processed for protein electrophoresis.

Protein electrophoresis and fluorography

Gill fragments from hsp induction experiments were placed in microcentrifuge tubes containing 300 μl of lysis buffer (32 m*M* Tris-HCl, pH 6.8; 2% sodium dodecyl sulfate (SDS) with 1 m*M* phenylmethylsulfonylfluoride (PMSF), a protease inhibitor, added immediately before use), and boiled for 2 min. Samples were then homogenized with a Teflon pellet pestle, boiled again for 5 min, and homogenized a second time. The homogenates were centrifuged at 16,000 $\times$ *g* in a microcentrifuge for 15 min. The supernatants were removed and the radioactivity, in counts per minute (CPM), of a 10-μl fraction of each sample was determined with a liquid scintillation counter; the remainder of the sample (about 200 μl) was stored at −20°C.

Proteins in the supernatants were separated by electrophoresis on 12% polyacrylamide gels in an SDS-buffer system (Laemmli, 1970). For ease of comparison and consistency, all of the samples (one gill fragment at each of the heat-exposure temperatures) from an individual mussel were analyzed on a single gel. Each sample was loaded in equivalent counts, approximately 600,000 CPM per lane. Gels were electrophoresed with 20 mA current for about 3.5 h, fixed for 1 h in an aqueous solution of 10% acetic acid and 30% methanol, and then incubated in EN^{3}HANCE (NEN) according to the manufacturer's protocol. Dried gels were exposed to film (Kodak X-OMAT) at −70°C for 15 h. After the film was developed, protein bands were analyzed with a densitometer (Molecular Dynamics), and the relative intensity and size of bands were quantified using ImageQuant software (Molecular Dynamics).

We measured the amount of newly synthesized protein (radioactivity) in a given band with a volume quantification procedure in ImageQuant, which calculated the intensity and the area of the band. The hsp bands were normalized relative to a non-heat-induced, strongly labeled 46-kDa protein band within the same sample lane to eliminate the variation in background labeling between samples. All comparisons of amounts of newly synthesized hsp70 were made relative to the amounts of newly synthesized 46-kDa protein ("relative amounts of

hsp70 synthesis"), for reasons given below. Normalization to the 46-kDa protein is appropriate because our objective was to estimate the temperatures at which enhanced synthesis of hsp70 relative to other size classes of proteins occurred, regardless of whether the change in relative synthesis rates was due to enhanced synthesis of hsp70 or to reduced synthesis of proteins, such as the 46-kDa protein, not induced at high temperatures. At the highest incubation temperatures, synthesis of proteins other than hsps was reduced. This observation was consistent with a defining characteristic of the heat-shock response: preferential synthesis of hsps over normal cellular proteins at abnormally high temperatures. Thus, the ratio of hsp70: 46-kDa protein could potentially increase as a result of an increase in hsp70 synthesis, a decrease in synthesis of the 46-kDa protein, or both. Our analysis of newly synthesized proteins therefore is appropriate for determining when hsp70 synthesis is induced by heat stress, even though this autoradiographic procedure cannot provide an estimate of the absolute amount of new hsp70 that is synthesized. Another reason for normalizing to the 46-kDa protein is the need to control for temperature effects on rate processes (Q_{10} effects). It could be argued that increases in hsp synthesis with rising temperature could simply be a consequence of Q_{10} effects on the rate of protein synthesis, and not a result of thermal induction of the heat stress response *per se.* By normalizing synthesis of hsp70 to synthesis of a non-heat-induced protein, we eliminated potential artifacts due to Q_{10} effects on synthesis rates.

For the graphical representations, but not for the statistical analyses, the relative amounts of hsp70 synthesized were normalized to the corresponding value at 10°C within each gel (*i.e.,* within each mussel), so that among the autoradiographs (*i.e.,* among the mussels) the relative intensities of hsp70 at 10°C were equivalent. This temperature is within the range of ambient seawater temperatures recorded at Strawberry Hill, and from preliminary experiments was shown not to induce synthesis of hsps in this population of *M. californianus.* By standardizing the relative intensity values for hsp70 to this common temperature, we could make graphical comparisons of the heat-shock response between sites and on a seasonal basis.

Hsp70 western blotting

Endogenous hsp70 levels in gill samples were quantified with immunoblotting techniques. For the field studies, gills dissected in the field in July 1993 and February 1994 were used; for the acclimation experiments in the laboratory, gills dissected in the field in May 1994 and from mussels acclimated to 10°C and 20°C for 7 weeks were used. Gill fragments dissected from mussels in the field and frozen immediately were homogenized in lysis buffer, following the same protocol used on samples for the induction experiment. Equivalent amounts of protein (5 μg) were electrophoresed on 7% or 7.5% SDS-polyacrylamide gels. This amount of protein was determined to be within the linear range of detection for the final immunochemical detection step (data not shown; see Hofmann and Somero, 1995). After electrophoresis, separated proteins were transferred from the gel to a nitrocellulose membrane *via* a semi-dry transfer blot apparatus (Owl Scientific). Transfers were conducted for 1.5 h at 115 mA with a transfer buffer containing 25 m*M* Tris base, 192 m*M* glycine, and 20% methanol. The nitrocellulose membrane was hydrated for 3 h prior to transfer, and the filter paper used to sandwich the membrane and gel was saturated in transfer buffer. After the transfer, the membrane was blocked overnight in blocking solution (5% nonfat dry milk, 0.02% thimerosal, in phosphate-buffered saline (PBS: 10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4)) and then rinsed three times for 10 min in PBS containing 0.1% Tween-20. The membrane was then sequentially incubated in solutions of rat monoclonal anti-hsp70 antibody (hybridoma 7.10; provided by Dr. Susan Lindquist of the University of Chicago), bridging antibody (rabbit anti-rat IgG; Vector Laboratories) and Protein A-horseradish peroxidase (HRP) conjugate (Bio-Rad). These methods are more fully described in Hofmann and Somero (1995). The enhanced chemiluminescence detection method (ECL detection reagents; Amersham) was used to visualize proteins that cross-reacted with the anti-hsp70 antibody. In order to compare multiple western blots, a biotinylated ECL molecular weight marker was run on each gel and visualized by adding a streptavidin-HRP incubation after the Protein A-HRP incubation step (see Hofmann and Somero, 1995). For individual western blots, the intensity of the ECL marker was used to standardize the intensities of the hsp70 bands. The chemiluminescent signal was detected according to the manufacturer's instructions, using pre-flashed Hyperfilm-ECL X-ray film (Amersham). Exposure times of the blot to the film were typically 5–15 s. Relative amounts of hsp70 were then quantified with densitometry and ImageQuant software.

Statistical analysis

SAS (SAS Institute Inc.) and SYSTAT (Systat Inc.) software programs were used for the statistical analyses. In induction experiments with field-acclimatized mussels, we compared the relative level of newly synthesized hsp70 in relation to incubation temperature, tidal height, and time of collection (*i.e.,* month). Using ANOVA techniques, incubation temperature (TEMP), collection time (MONTH), and tidal height of collection site (HEIGHT) were designated as factors, or main effects (*i.e.,* independent variables), and the relative level of newly synthe-

sized hsp70 was designated as the response variable (*i.e.*, dependent variable). Because individual mussels were divided into separate gill fragments, *subunits*, in the induction experiments, a split-plot ANOVA was used (Sokal and Rohlf, 1981). MONTH and HEIGHT were *unit* (whole mussel) level effects, and TEMP was a *subunit* (gill fragment) level effect. A variable to designate "among whole mussel variation" (MUSS) was also included in the analysis. In this case, the null hypothesis was that the variation in hsp70 synthesis was random, and not due to TEMP, MONTH, or HEIGHT. Because the relative level of newly synthesized hsp70 was calculated as a ratio, and because exploratory data analyses revealed unequal variances, these data were log-transformed prior to running the analysis. A similar model was used to analyze the results of the hsp induction experiments with the laboratory-acclimated mussels. We were interested in comparing the relative level of newly synthesized hsp70 in relation to incubation temperature and acclimation treatment (pre-acclimation (field), 10°, 13°, 17°, and 20°C acclimation). Incubation temperature (TEMP) and acclimation treatment (ACCLTRT) were the independent variables, and the relative level of newly synthesized hsp70 was the dependent variable.

ANOVA was also used to analyze the results of the hsp70 western blotting. For the field studies, the relative level of endogenous hsp70 was the dependent variable, and collection season, tidal height, and collection time were the independent variables. For the laboratory acclimation studies, the relative level of endogenous hsp70 was the dependent variable, and acclimation treatment was the independent variable (Sokal and Rohlf, 1981). The Bonferroni correction procedure (Systat) was used for *post hoc* multiple comparisons (Schlotzhauer and Littell, 1987).

Results

Tissue temperatures of mussels in the field

Internal tissue temperatures of attached mussels during emersion at low tide were recorded seasonally at Strawberry Hill, using mussels from both high- and low-intertidal sites. Tissue temperatures in May and July ranged from about 9° to 28°C. Tissue temperatures on a day in July 1993 are shown in Figure 1 (top). Tissue temperatures were above 18°C for 2.5 h during emersion at the high site and for less than 30 min at the low site. Temperature data from a day in May 1993 (Fig 1, bottom) show that tissue temperatures were above 18°C for about 3.5 h at both sites. The temperatures of the low-site animals reached slightly higher peak values than conspecifics at the high site.

These data indicate the potential range and variability in tissue temperatures, but because measurements were only made over discrete time intervals around low tide, and because weather conditions (*e.g.*, wind, cloud cover) can fluctuate immensely on a daily basis, the data should not be interpreted to represent general seasonal patterns. In addition, these data show the unpredictable nature of the temperature ranges experienced by mussels on any given day. The time of day of the lowest low tide each day during spring tides varies seasonally; in the fall and winter the extreme low tides are in the early evening and night, and in the spring and summer the extreme low tides are in the early to mid morning (*1995 Tide Tables for the Pacific Coast of North and South America*, NOAA, US Dept. of Commerce).

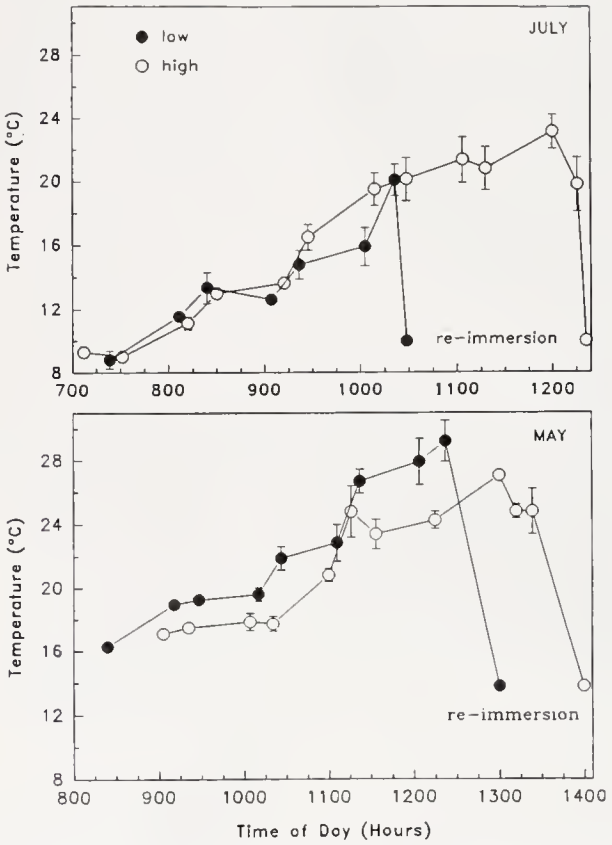

Figure 1. *In situ* tissue temperatures of *Mytilus californianus* in relation to time of day over a single low-tide emersion period in July (top) and May (bottom) 1993, at high- and low-intertidal sites at Strawberry Hill, Oregon. Each point represents the mean temperature of 5 mussels. Error bars are ±1 standard error of the mean.

Heat-shock-protein induction profiles

General patterns of newly synthesized proteins in field-acclimatized mussels. Experiments on induction of heat-shock proteins were conducted on freshly field-collected mussels in February, March, May, June, and August of 1994. In most cases, direct visual analysis of labeled protein bands revealed a clear profile of hsp induction: as

incubation temperature increased, the relative intensities of bands representing newly synthesized proteins changed markedly, with bands of molecular mass corresponding to major classes of heat shock proteins showing the strongest increase in intensity (Fig. 2). The most prominent bands of heat-inducible protein were of apparent M_r 68–74 kDa and 31.5 kDa—bands that we assume to represent heat shock proteins of the 70-kDa and 30-kDa classes. These bands were faint or absent at temperatures below 20°C, and exhibited a marked increase in intensity and width at temperatures of 23°C and higher (Fig. 2). The appearance of two strong bands with M_r values near 70 kDa indicated *de novo* synthesis of more than one hsp70 isoform (Fig. 2). Other protein bands, whose synthesis was not induced by heat, were consistently present in all samples and at all temperatures tested. Most notable were two bands of 39 and 46 kDa (Fig. 2). However, synthesis of these non-heat-induced proteins decreased at 25° and 28°C, temperatures at which hsp synthesis remained strong.

Seasonal and tidal-height patterns in hsp70 induction profiles. In February, in mussels collected from a mid-intertidal site, there was a significant increase in the relative amount of hsp70 synthesis at 23°, 25°, and 28°C as compared to synthesis at lower temperatures (Fig. 3; ANOVA, $P < 0.001$ in all cases). Between 23° and 28°C there was a twofold increase in the relative amount of hsp70 synthesized, and synthesis levels at 28°C were about four times higher than at 10°, 17°, and 20°C (Fig. 3). The importance of normalizing hsp70 synthesis to the 46-kDa protein to determine induction temperatures is illustrated by comparing data in Figures 2 and 3. Although Figure 2 appears to show an increase (induction) in hsp70 synthesis at 20°C, when the 70-kDa band is normalized to the 46-kDa band (Fig. 3) no change in the relative amount of hsp70 synthesis is found until 23°C.

Beginning in March, hsp induction experiments were performed on mussels collected at high- and low-intertidal sites, to examine site location and seasonal effects on patterns of hsp synthesis. The overall trends in the March and February specimens were similar: a strong increase in relative synthesis of hsps of both general size classes occurred at 23°C, and hsp70 levels were about fourfold higher at 28°C than at temperatures of 20° and lower (Fig. 4). In March, the relative magnitude of the response was somewhat elevated in the low-intertidal

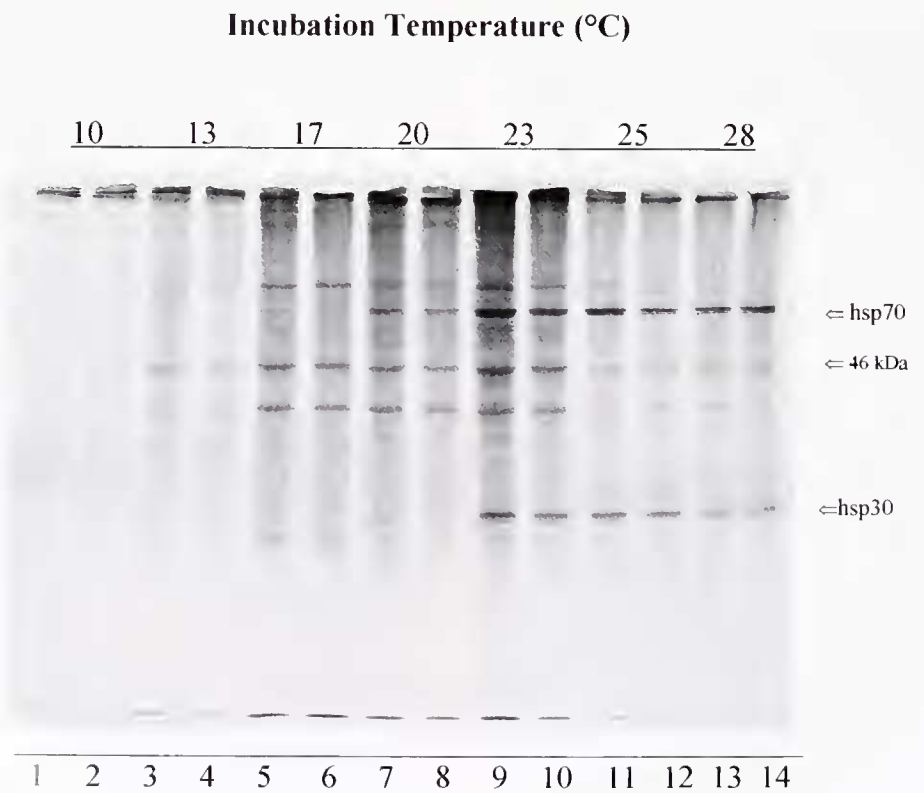

Figure 2. Autoradiograph from an experiment on the induction of heat shock proteins in *Mytilus californianus*. Fragments of gill tissue from a single individual (collected in February 1994) were radiolabeled with a mixture of ^{35}S-methionine and ^{35}S-cysteine for 2 h. Samples were loaded on a 12% polyacrylamide gel in duplicate; approximately 600,000 CPM per lane were loaded. Lanes 1–2, 10°C; lanes 3–4, 13°C; lanes 5–6, 17°C; lanes 7–8, 20°C; lanes 9–10, 23°C; lanes 11–12, 25°C; lanes 13–14, 28°C.

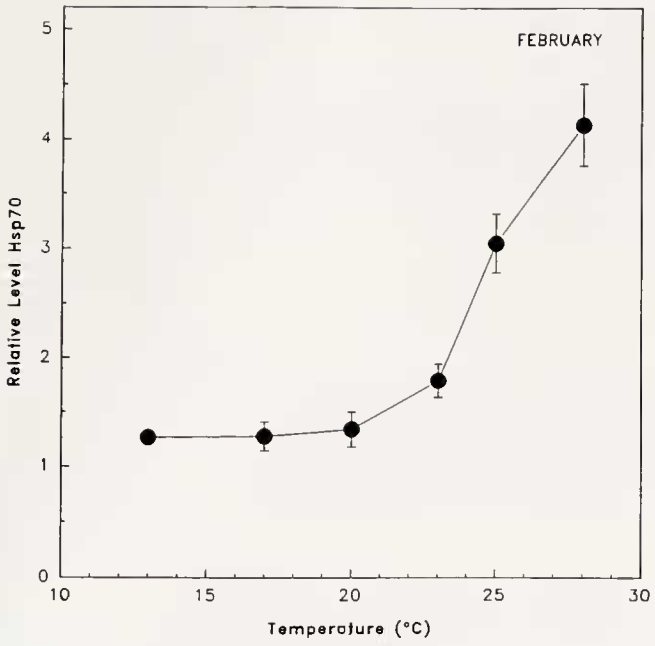

Figure 3. Hsp induction in gill of *Mytilus californianus* in relation to incubation temperature. Mussels were collected in February 1994 from a mid-intertidal site at Strawberry Hill. Each point represents the mean relative level of newly synthesized hsp70 of 6 mussels; error bars are ±1 standard error of the mean.

mussels as compared to the high-intertidal mussels at all temperatures, although this difference was not significant (Fig. 4). The induction profiles in the May samples are similar to those in February and March, although there was an overall reduction in the magnitude of the response. The June samples showed a further dampening of the induction response (note that 13° and 17°C were not tested in the June collection; Fig. 4), and in the August samples there was no longer a sharp induction profile in either the high or low samples (note that 13°C was not tested; Fig. 4).

A split-plot ANOVA indicated that incubation temperature (TEMP, $P < 0.0001$), collection month (MONTH, $P < 0.0007$), and the interaction term between temperature and collection month (MONTH * TEMP, $P < 0.0001$) were statistically significant in explaining variation in relative amounts of new synthesis of hsp70. There was significant individual variability in hsp70 expression among individual mussels (MUSS (MONTH * HEIGHT), $P < 0.0001$), but the tidal height of the collection site was not significant in the model (HEIGHT, $P < 0.7470$).

Hsp70 synthesis patterns in laboratory-acclimated mussels. Freshly collected (= pre-acclimation) specimens of mid-intertidal *M. californianus* collected in May 1994 exhibited an hsp70 expression pattern (data not shown) similar to that observed with high- and low-in-

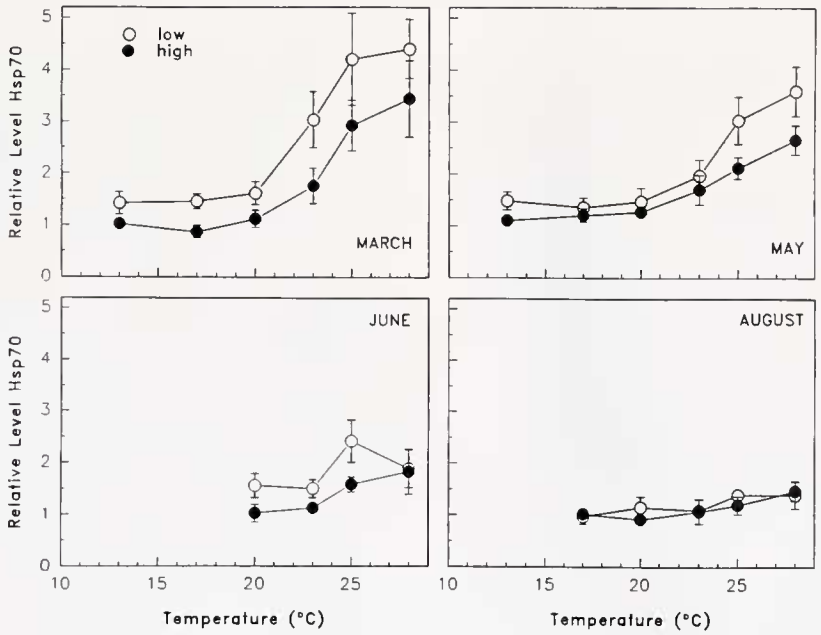

Figure 4. Hsp70 induction in gill of *Mytilus californianus* in relation to incubation temperature in field-collected mussels in March, May, June, and August 1994, from high- and low-intertidal sites at Strawberry Hill. Points represent means: March low, $n = 5$; March high, $n = 4$; May low, $n = 8$; May high, $n = 8$; June low, $n = 5$; June high, $n = 4$; August low, $n = 3$; August high, $n = 3$. Error bars are ±1 standard error of the mean.

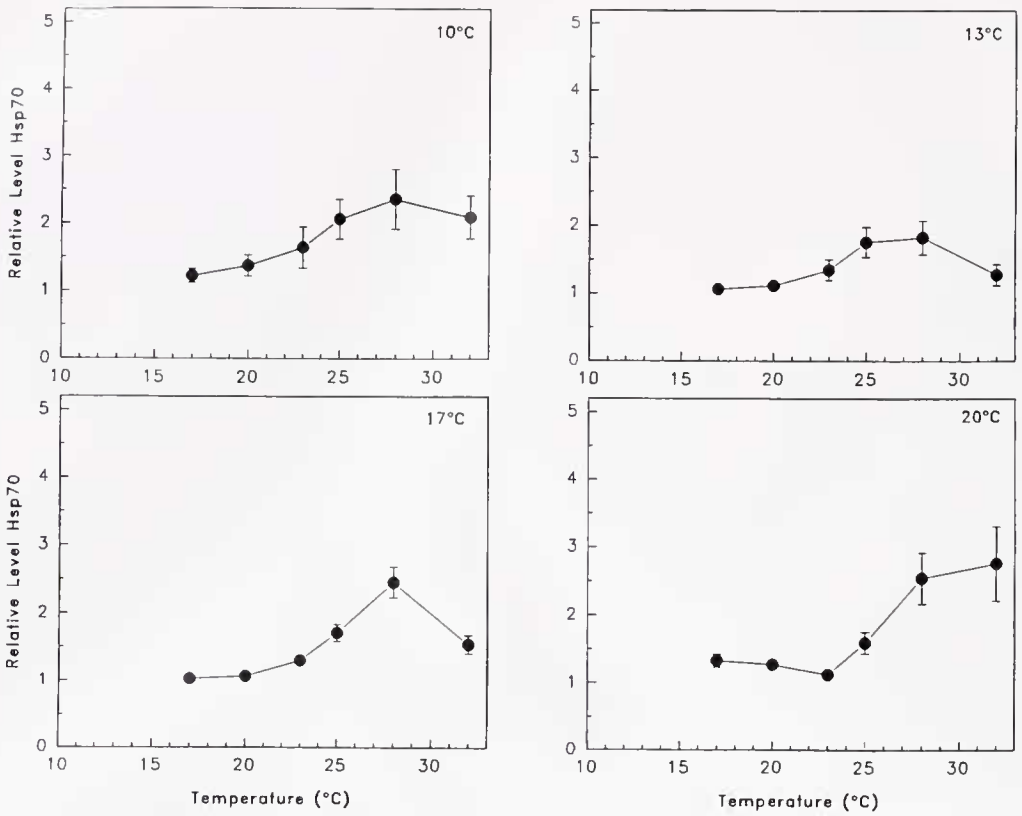

Figure 5. Hsp70 induction in gill of laboratory-acclimated *Mytilus californianus* in relation to temperature. Mussels were collected in May 1994 from a mid-intertidal site at Strawberry Hill and acclimated in the laboratory for 7 weeks. Each point represents the mean relative level of newly synthesized hsp70; error bars are ±1 standard error of the mean. Sample sizes were 10°C-acclimation, $n = 5$; 13°C-acclimation, $n = 6$; 17°C-acclimation, $n = 7$; 20°C-acclimation, $n = 7$.

tertidal mussels from the same month (Fig. 4): amounts of newly synthesized hsp70 were relatively constant and low between 10°C and 20°C, but between 20°C and 25°C there was a sharp increase in synthesis of hsp70. The amounts of newly synthesized hsp70 at 25°C and 28°C were more than three times the levels at 10°C and 13°C.

A comparison of these data with results from the temperature-acclimated mussels (Fig. 5) revealed several differences. Overall, the relative magnitude of the amount of newly synthesized hsp70 was reduced in the mussels acclimated to 10°, 13°, and 17°C, when compared to the pre-acclimation mussels (compare May data in Fig. 4 with data in Fig. 5). In all but the 20°C-acclimated specimens, the relative amount of new hsp70 synthesis was maximal at 28°C and decreased at the highest incubation temperature, 32°C. Protein synthesis in the 20°C-acclimated mussels was more resistant to high temperature, and no decrease in hsp70 synthesis was noted at 32°C. Acclimation had no consistent effects on the temperature at which a significant increase in hsp70 synthesis first occurred.

The split-plot ANOVA model showed that acclimation treatment (ACCLTRT, $P < 0.0001$), temperature (TEMP, $P < 0.0001$), and the interaction between these two factors (ACCLTRT * TEMP, $P < 0.0001$) explained a significant portion of the variation in relative levels of newly synthesized hsp70. This model also shows that the variation in response among individual mussels was significant ($P < 0.0052$).

Endogenous hsp70 levels: constitutive and heat-induced isoforms

Western blotting with a rat monoclonal anti-hsp70 antibody was used to measure the relative amounts of hsp70 present in field-acclimatized *M. californianus* over the low-tide emersion period, in high- and low-intertidal populations, and in summer- and in winter-acclimatized specimens (Fig. 6). For quantification and comparison, the hsp70 bands were divided into two groups of isoforms; a lower molecular mass group (LMM-hsp70, apparent M_r: 66–68 kDa) and a higher molecular mass group (HMM-hsp70, apparent M_r: 69–73 kDa). These two molecular-mass classes may include both constitutively synthesized isoforms of 70-kDa chaperones (hsp70 cognates = hsc70) and heat-induced isoforms (hsp70s)

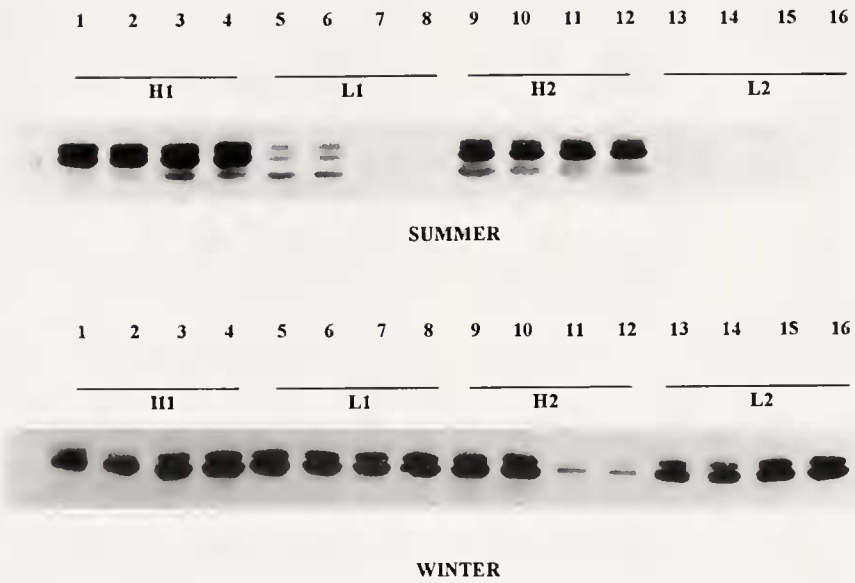

Figure 6. Western blot analysis of hsp70 isoforms in gill tissue of summer- and winter-acclimatized *Mytilus californianus*. Mussels were collected at high- and low-intertidal sites just as the water was receding (H1 and L1, respectively) and just prior to re-immersion (H2 and L2, respectively). Lanes were loaded with equal amounts of protein (5 μg), and immunodetection was performed using ECL reagents. Two samples from each site were loaded in duplicate (*e.g.*, lanes 1 and 2 are the same sample). It should be noted that one individual (H2 in Winter) displayed significantly lower levels of endogenous hsp70; the reason for this difference is unknown.

because this anti-hsp 70 antibody reacts with both types of 70-kDa isoforms.

As shown in Figure 7, there were no significant differences in hsp70 levels over a single low-tide emersion period in either the high- (H1, H2) or low- (L1, L2) intertidal sample, in either the summer or winter collections. However, distinct seasonal and tidal height differences, both in the quantity and banding pattern of hsp70 proteins, were observed (Fig. 7). The relative amount of LMM-hsp70 isoforms was significantly greater in the high-intertidal mussels than in the low-intertidal mussels in the summer collection ($P < 0.01$). In the winter, there was no significant difference in the amount of LMM-hsp70 in the gill of high- and low-intertidal mussels (Fig. 7a). Within the high-intertidal site, there were significantly higher levels of LMM-hsp70 isoforms in the summer than in the winter ($P < 0.01$). Within the low site, there were no significant seasonal differences.

Consistent with the patterns shown by LMM-hsp70, the amount of HMM-hsp70s in summer was significantly higher in the high-intertidal mussels than in the low-intertidal mussels (Fig. 7b; $P < 0.01$). However, within the high-intertidal site, there were no significant differences seasonally, and within the low intertidal site, the amount of HMM-hsp70 was higher in winter than in summer.

Using western analysis, we also compared hsp70 levels in the pre-acclimation group of field-collected mussels (May 1994), and in the laboratory-acclimated mussels after 7 weeks of acclimation to either 10° or 20°C (data not shown). There were no significant differences among any of these three treatment groups for either the LMM or HMM forms of hsp70.

Discussion

In situ *tissue temperatures and expression of heat-shock proteins*

The tissue temperatures recorded *in situ* during emersion at all seasons frequently reached levels at which increased synthesis of hsp70 was observed in the *in vitro* labeling studies (Figs. 1–4). These observations, in concert with the seasonal and tidal-height variations observed in endogenous levels of hsp70 (Figs. 6 and 7), show that the heat-shock response is likely to be induced in mussels at all seasons, but particularly during summer when low tides typically occur during midday. Because the intensity of thermal stress is due to the product of temperature multiplied by duration of exposure, mussels in the higher regions of the intertidal zone experience greater heat stress, even though the absolute tissue temperatures reached during emersion on hot days may not differ between high- and low-intertidal individuals (Fig. 1). Other data support our conclusion that tissue temperatures of mussels frequently become high enough to induce the heat-shock response. Elvin and Gonor (1979) recorded tissue temperatures ranging from 0° to 34°C in *M. californianus* on the Oregon coast. Hofmann and

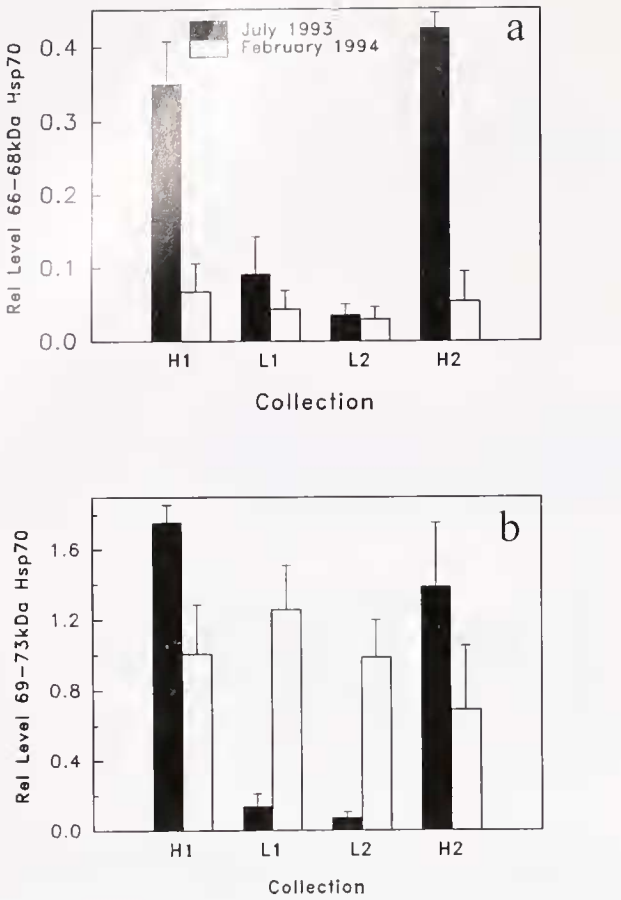

Figure 7. Mean relative level of low molecular mass (66–68 kDa) hsp70 isoforms (a) and high molecular mass (69–73 kDa) hsp70 isoforms (b) in *Mytilus californianus* gill in relation to intertidal collection site, over a low-tide emersion period in summer and in winter. H and L designate samples from high and low sites, respectively; 1 and 2 designate samples collected at the beginning and end, respectively, of the period of emersion. Error bars are ±1 standard error of the mean (n = 5).

Somero (1995) reported tissue temperatures as high as 32°C during emersion of *M. trossulus* during midday low tides in coastal intertidal sites on San Juan Island, Washington. These data on body temperature, taken in conjunction with the hsp induction profiles presented here and in other studies of intertidal invertebrates (Hofmann and Somero, 1995, 1996), highlight how important stress-protein expression may be to eurythermal ectotherms, such as intertidal invertebrates, for tolerance of fluctuations in environmental temperature and the development of seasonal thermotolerance (Coleman *et al.*, 1995).

Seasonal patterns: induction profiles and endogenous levels of heat-shock proteins

The results of the *in vitro* labeling experiments showed that patterns of hsp expression varied significantly over the 7-month period of the field experiment (Figs. 3, 4; $P < 0.0007$). Two observations illustrate the plasticity of the stress response in field organisms. First, there was significant seasonal variation in the induction profiles with respect to induction temperatures and the magnitude of new synthesis of hsp70 isoforms (Figs. 3 and 4). In gill from mussels sampled in February and March, hsp70 synthesis was strongly induced at temperatures greater than 20°C. By May, the induction response was dampened, and this trend continued in June and August. Thus, in what may appear to be a paradoxical trend, the amount of new synthesis of hsp70 was lowest in the months when average temperatures during emersion were likely to be highest.

Few other studies have examined seasonal changes in the heat-shock response. In an estuarine goby fish, *Gillichthys mirabilis*, the induction temperature for synthesis of hsp90 increased from 28°C in winter-acclimatized fish to 32°C in summer-acclimatized fish (Dietz and Somero, 1992). The plasticity observed in invertebrates (this study) and fishes (Dietz and Somero, 1992) in the induction of hsp synthesis indicates that the regulation of hsp expression is not a genetically fixed characteristic of an organism, but is instead a trait subject to acclimatization.

The second type of plasticity noted in the field-acclimatization experiment was the strong effect of season on endogenous levels of hsp70 isoforms (Figs. 6 and 7). During summer, when the highest body temperatures during emersion are likely, the levels of the LMM isoforms of hsp70 were significantly elevated over levels measured in winter mussels in the high-intertidal location, but not at the lower site (Fig. 7; see below). For the HMM isoforms of hsp70, summer levels were significantly elevated in mussels from the high-intertidal site as compared to levels in mussels from the low-intertidal site (Fig. 7). The seasonal changes noted in endogenous levels of hsp70 isoforms may provide a partial explanation (but see below) for the seemingly paradoxical result found in the induction experiments—namely, the dampened induction response noted in summer specimens. The higher levels of hsp70 isoforms maintained in summer may be a type of "anticipatory" adaptation to the likelihood of increased thermal stress in these months. Higher endogenous levels of hsps may preclude the need for induction of new hsp synthesis in concert with emersion during the tidal cycle.

Tidal height patterns: induction profiles and endogenous levels

No significant differences were found in the induction profiles of mussels from high- and low-intertidal sites (Fig. 4), even though the endogenous levels of hsp70 isoforms in the two groups were significantly different (Fig. 7). In summer, the levels of LMM and HMM hsp70 were

significantly higher in mussels from the high site than in individuals from the low site, an observation consistent with greater amounts of thermal stress in the high intertidal individuals. In winter, for both isoforms of hsp70, high- and low-site mussels had the same hsp70 levels. These data show that the temperatures at which new synthesis of hsp70 isoforms is induced are not established entirely by the endogenous levels of hsp70. One reason for the observed lack of a consistent correlation between endogenous levels of hsp70 isoforms and induction temperature may be that the data from the western analysis includes both constitutively expressed and heat-induced isoforms of hsp70, whereas the data from *in vitro* labeling primarily represents new synthesis of the heat-inducible isoforms. A clearer understanding of the correlation between endogenous levels of an hsp and the induction temperature for new hsp synthesis thus requires the ability to to distinguish between constitutively expressed and heat-induced isoforms, something our antibody analysis did not do.

Studies with other intertidal invertebrates have also observed differential expression of stress proteins as a function of intertidal location. The limpet *Collisella scabra,* which occurs relatively high in the intertidal zone, expressed more isoforms of hsp70 and hsp60 than a congener, *C. pelta,* which occurs lower in the intertidal in shaded surge channels (Sanders *et al.,* 1991). In the sea anemone *Anemonia viridis,* intertidally occurring individuals have higher constitutive levels of a low molecular weight hsp than do anemones from subtidal collections (Sharp *et al.,* 1994). In *M. trossulus,* intertidal populations had higher endogenous levels of hsp70 than subtidal populations (Hofmann and Somero, 1995).

Acclimation patterns: induction profiles and endogenous levels of heat-shock proteins

The acclimation experiments were conducted to determine whether the seasonal acclimatizational effects noted in induction profiles and endogenous levels of hsp70 could be mimicked through cold- and warm-acclimation in the laboratory. That is, the acclimation experiments were designed to test the hypothesis that changes in temperature alone were responsible for seasonal acclimatization of the heat-shock response. Our data refute this hypothesis. The induction profile data (Fig. 5) show that the warm- and cold-acclimated mussels had indistinguishable heat-shock responses. Both acclimation groups exhibited a relatively attenuated response, with maximal increases in hsp70 synthesis of no more than about 2.5-fold, in contrast with the greater than 4-fold increases seen in winter-acclimatized mussels (Figs. 3 and 4). Similarly, no significant differences were found between pre-acclimation, 10°-, and 20°C-acclimated groups in the endogenous levels of LMM and HMM hsp70 isoforms.

The differences that distinguish the results of the acclimation and acclimatization experiments with *M. californianus* raise caveats about the design and interpretation of heat-shock experiments. Particularly for sessile intertidal species like *M. californianus,* which encounter a variety of emersion-related stresses, including those due to desiccation and anaerobiosis, it may be unrealistic to assume that temperature is the only, or even the primary, stress that necessitates the heat-shock (stress) response. A common signal for hsp induction is the appearance in the cell of unfolded proteins (Ananthan *et al.,* 1986; Parsell and Lindquist, 1993). In addition to thermal stress, changes in intracellular ionic strength and pH during emersion could affect protein structure and, thereby, induce the synthesis of heat-shock proteins. Because the mussels in the acclimation experiment were continuously submerged, stresses due to desiccation and anaerobiosis were not present. Therefore, acclimation to a single environmental variable, for example, temperature, may not lead to a heat-shock response that mimics the response occurring under *in situ* conditions.

General conclusions

Synthesis of stress proteins, like all protein synthesis, is expensive in terms of the energy budget of an animal (Creighton, 1993). Hawkins (1991) has estimated the costs of protein synthesis to constitute 20%–25% of the energy budget of the bay mussel, *Mytilus edulis.* This cost represents an additional energy burden because stress proteins do not directly contribute to increases in growth or reproduction, and because under stress conditions they may be synthesized preferentially, such that other proteins critical for the normal functioning of the organism are either synthesized at reduced rates or not synthesized at all. Furthermore, the function of stress proteins may require considerable ATP turnover; refolding of a protein may consume in excess of 100 ATP molecules (Creighton, 1991; Martin *et al.,* 1991; Parsell and Lindquist, 1993). The extent to which different intensities of thermal stress found in high- and low-intertidal sites impact protein synthesis and growth is not known. However, significant differences in growth rates (Menge *et al.,* 1994) and in capacity for protein synthesis as indicated by RNA:DNA ratios (Dahlhoff and Menge, 1996) have been found between mussels in different tidal locations at Strawberry Hill. Although these height-related differences may be due in large measure to variation in food availability from site to site (Dahlhoff and Menge, 1996), other explanations are possible. The data also are consistent with the conjectures that the heat shock response can exact a measurable toll on the energy budgets of organisms (Krebs and Loeschcke, 1994; Coleman *et al.,* 1995), and that thermal damage to proteins could play a key role in defining the habitat ranges of marine species (Somero, 1995).

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